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Molecular survey of helminths infecting ground dwelling birds in the grouse subfamily Tetraoninae

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An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Biology

By

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Under the mentorship of Dr. Stephen Greiman

ABSTRACT

Alaskan grouse and ptarmigan are a significant game bird in Alaska and are found to harbor a fairly diverse helminth parasite fauna (flatworms (tapeworms, flukes) and roundworms (nematodes)). Interestingly, these intestinal parasites, particularly cestodes, may make these birds more susceptible to predation. Unfortunately, there is limited information available on the helminth fauna of Alaskan grouse including no molecular DNA surveys.

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Introduction

Parasitism is a complex life history trait used by over half of global biodiversity. Many parasites, both ecto- and endo- display an aggregated distribution on or within their host species (i.e. many hosts with no or few parasites and few hosts with many). Parasites can alter host behavior, change predator prey models, and are indicators of a healthy ecosystem (Hudson et al. 2006). Additionally, it is common for hosts to experience polyparasitism; several different parasite species on or within a single host individual (Holmstad & Skorping 1998). These parasitic communities can cause rather detrimental effects to their hosts including: decreases in reproduction, fecundity, size and overall fitness (Holmstad et al. 2005). Parasite communities can vary greatly depending on geographic location and/or host characteristics, as several studies have shown a positive correlation in the prevalence of helminths related to host age and habitat (Davidson et al. 2011). Previous studies have shown the presence of parasites have caused declines in the numbers of ground dwelling birds in Norway (Holmstad et al. 2005).

Alaskan grouse and ptarmigan are a significant game bird in Alaska and are found to harbor a fairly diverse helminth parasite fauna (flatworms (tapeworms, flukes) and roundworms (nematodes)). Interestingly, these intestinal parasites, particularly cestodes, may make these birds more susceptible to predation. As a game bird, ptarmigan and grouse are typically hunted by canine. It has been shown that when under the stress of being hunted by dogs, a replica of natural predation, ground dwelling birds harbor more parasites (Isomursu et al. 2008). Unfortunately, there is limited information available on the helminth fauna of Alaskan grouse including no molecular DNA surveys.

Even though there has never been a molecular DNA survey of the parasites found in Alaskan ground dwelling fowl, there have been morphological based surveys. These works have shown parasites found from the Nematoda and Platyhelminthes (classes: trematoda and cestoda) phyla. *Ascaridia compar,* a species of nematode often observed within the small intestine of Alaska ptarmigan, are identifiable by the number of post-anal papillae found on male specimens. *Trichostrongylus tenuis* is a species of nematode found in the ceca of willow ptarmigan collected on the Alaskan Peninsula and Talkeetna Mountains. *Microfilaria lagopodis* is a blood nematode found to infect willow ptarmigan in Alaska and Holland. *Leucochloridium carus* is a digenean that is prevalent in high numbers in spruce grouse. *Brachylaima fuscata* is the most observed species of digenean in Alaska. *Brachylaima fuscata* has been collected from Anaktuvuk pass, Kotzebuem and the Anchorage area from several different species of birds including: ruffed grouse, sharp-tailed grouse, and spruce grouse. *Haploparaxis galli* is a cestode that was first found in rock ptarmigan, but later located in willow ptarmigan; *H. galli* has been located in Tulugak Lake, Fairbanks, Nome, and Kotzebue. *Davainea proglottina* is a common species of cestode that has been observed in Tulugak Lake, Fairbanks, and the Kenai Peninsula. *Davainea proglottina* can easily be identified by the double row of rostellar hooks and large number of testes. *Railietina urogalli* is a species of cestodes found in the willow ptarmigan and red grouse. *Railietina uogalli* has been collected from Talkeetna Mountains, Lake Schrader, Anaktuvuk Pass, and Anchorage. *Rhabdomeira mullicollis* is a species of cestode that is often found in the small intestine of rock ptarmigan (Babero 1953).

Not only do helminth parasites cause declines in the numbers of ground dwelling birds, it is thought that vector-borne parasites, such a filariid nematode, cause even higher mortality rates. A study was conducted over a 9-year period looking at the "freeze or flee" behaviors in willow ptarmigan (Holmstad et al. 2006). This study showed that all vector-borne parasites undoubtedly had significant effects on the host response to freezing or fleeing. All hosts harboring vector-borne parasites had a tendency to freeze with potential threat instead of fleeing. These effects could be evolutionary advantageous to parasites because immobile hosts are easier prey for blood-sucking insects (Holmstad et al. 2006).

The present study aimed at developing baseline data on the diversity of intestinal and subcutaneous (filariid nematodes) helminth infections in grouse using morphological and molecular (DNA) approaches. These data can then be used to better understand the changes in helminth community structure given current environmental instability. We looked at the prevalence of parasites in 83 ground dwelling fowls collected from various sites in Alaska; we found that 89% of gallids collected were infected with one or more parasite taxa. We also aimed to look at the presence or absence of microfilaria located within these host species. Common practice was to test for blood parasites, including microfilaria, through blood smears from peripheral blood samples; however, this may not be the best method in the case of microfilaria because they tend to be present within deeper circulation (Holmstad et al. 2003).

Methods

Several different species of Alaskan ptarmigan and grouse were collected in various parts of Alaska. Each specimen was collected between May and September 2017

by ballistic capture. Whole frozen birds and bird intestines in whirlpacks (frozen) were provided from colleagues at the Alaska Science Center in Anchorage (United States Geological Survey). The intestines, cloaca, liver and kidneys of 83 Alaskan grouse and ptarmigan (*Lagopus lagopus*, *Falcipennis Canadensis*, *Lagopus muta*) were examined for the presence of helminths (nematodes, cestodes, digeneans) through necropsy using a dissecting microscope. Helminths (nematodes, cestodes, digeneasn) found were preserved in 80% ethanol.

A total of 564 blood/muscle DNA extracts were screened for the presence of *Splendidofilaria* spp. DNA using a newly developed real-time Taqman PCR assay *Bonasa umbellus* (n=23), *Lagopus leucura* (n=23), *Tympanuchus phasianellus* (n=12), *Dendragapus fuliginosus* (n=8), *Falcipennis canadensis* (n=139), *Lagopus lagopus* (n=223), and *Lagopus muta* (n=136). Real Time PCR using TaqMan Probes targeting the 18S rDNA gene will be used to test for the presence of filarial nematodes. A total of 22 flies were removed from 16 *B. umbellus*, 4 *F. canadensis*, 1 *L. lagopus*, and 1 *L. muta* and screened for the presence of microfilaria in order for determination of possible vector.

Adult worm extraction

Fragmented adult *Splendidofilaria* sp. were obtained from a single *F. Canadensis* individual. DNA extraction of two fragments was completed following the protocol by (Tkach and Pawlowski 1999). In short, DNA extraction of an ethanol-fixed parasite was performed by cutting an approximately 2mm tissue sample from the middle of nematode. The tissue sample was transferred to a microcentrifuge tube and all extra ethanol was removed. Microcentrifuge tube was placed on heat block for 15 minutes to evaporate all

remaining ethanol. 95µl of digestion buffer, 95µl of H_2O, and 10µL of Proteinase K was added, vortexed, and placed on heat block overnight. 200µL of isopropanol was added to microcentrifuge tube, vortexed, and placed in the freezer for a minimum of 2 hours. Microcentrifuge tube was then centrifuged for 15 minutes at 15,000 RPM or max speed. Supernatant was removed from microcentrifuge tube using a fine pipette, washed with 200µL of 70% ethanol, and vortexed gently. The microcentrifuge tube was centrifuged again at max speed for 15 minutes. Ethanol was removed from microcentrifuge tube and another wash was performed with 200µL of 70% ethanol. The microcentrifuge tube was gently vortexed, centrifuged for 5 minutes, and all ethanol was removed. The microcentrifuge tube was then placed on the heat block for 15-20 minutes to insure all ethanol has evaporated. 45µL of H_2O was added to microcentrifuge tube to resuspend the DNA pellet. The microcentrifuge tube was vortexed gently and placed in a dark drawer for 2 hours. After the 2-hour period, the microcentrifuge tube was placed in the freezer.

Fly extraction

Flies were collected from three different species of birds, *B. umbellus*, *F. canadensis*, *L. lagopus*, and *L. muta*. Extractions were completed using the above detailed guanidine thiocyanate method (V. Tkach and Jan Pawlowski 1999).

PCR amplification

Polymerase chain reaction method (PCR) was used to target the 18S rRNA gene in the filarial nematode by using 12.5µL of Promega master mix, 8.5µL of H_2O, 1µL of sple-86f, 1µL sple-461R, and 2µL of DNA from the filarial nematode was added to a microcentrifuge tube. PCR was also used to target the 16S region of DNA in the filarial

nematode by using 12.5µL of Promega master mix, 8.5µL of H_2O, 1µL of sple-CO1f, 1µL sple-CO1R, and 2µL of DNA from the filarial nematode was added to a microcentrifuge tube. PCR amplification was run using a SimpliAmp Thermal Cycler. The PCR was cleaned under the hood by using 5µL DNA and 2µL ExoSapit enzymatic cleanup. The primers were tested to see if they annealed to the filarial nematode DNA by gel electrophoresis.

TaqMan probe development and usage

Samples were screened using Real Time PCR and a TaqMan probe to study for the presence or absence of microfilaria. We amplified a 50-100 base pair fragment in order to develop primers using the CO1 mtDNA gene. We insured our newly designed TaqMan probe is specific to the microfilaria, Splendidofilariae, found to infect gallids in Alaska by comparing the CO1 mtDNA alignment to other species of microfilaria, to include *Dirofilaria repens*, *Filariodea* sp., *Loa loa*, *Onchocerca flexuosa*, *Setaria_tundra*, and *Setaria digitate* (FIGURE 1). Real Time PCR was tested using 7.5µL of TaqMan master mix, 4.25µL of H_2O, 2µL of DNA, and 1.25µL of primer/probe mixture. Real Time PCR was ran by using a StepOne Real Time PCR machine.

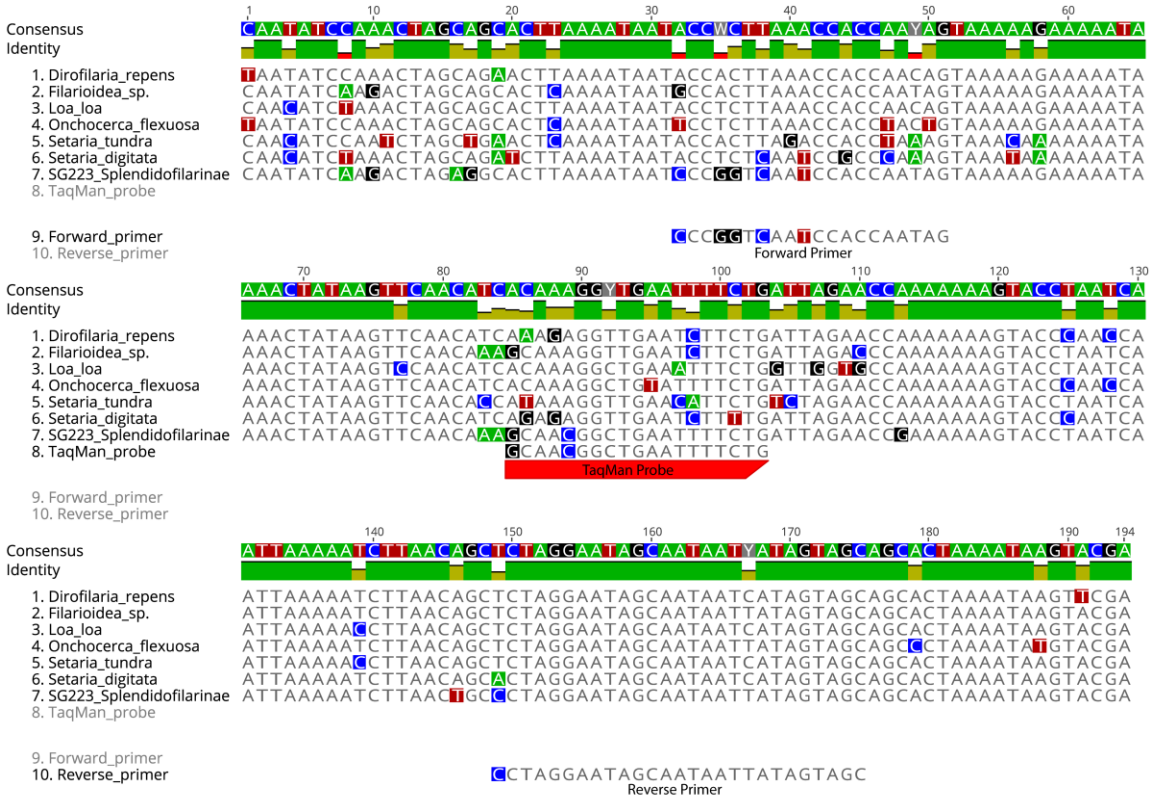

Figure 1: COI mtDNA alignment of Filariid nematodes. SG223_Splendidofilarinae was obtained from our study and found to infect gallids in AK. Red box indicates our newly designed TaqMan probe for real-time PCR screening of blood and tissue samples.

Results

Out of a total of 73 gallids, 65 were infected by one or more parasites, equating to 89% of birds being infected. Willow ptarmigan showed the highest prevalence of infection; however, this could have been due to the higher number of willow ptarmigan sampled compared with other bird species. Below, Table 1 shows the total number and percentages of all parasites found within each host species. Figure 7 shows the three significant location sites of samples collected for GI necropsy. The most abundant helminth taxa present were in the class Cestoda, 80.6% of all gallids infected; specifically, *Davainea proglottina* (69.4%) and *Hymenolepidae sp.* (12.5%). There was also a high prevalence of species within the class Trematoda, 37.5% of all gallids

infected; specifically, *Leucochloridium variae* (25%) and *Brachylaima fuscata* (27.8%). Taxa in the phylum Nematoda were also prevalent, with 15.3% of all gallids infected; specifically, *Ascaridia compar* (6.9%) and *Capillaria sp.* (9.7%). It is interesting to note that all *Capillaria sp.* present were located in the large intestine instead of the small intestine where they are normally found.

Davainea proglottina has been found in various parts of the world including: most countries in Europe, North America, South America, Africa, India, and Australia (Abbou, 1958). An important identifying characteristic in *D. proglottina* is an acetabula scolex with rostellar hooks; as you can see in Figure 2. When there are limited numbers of *D. proglottina* infecting the host there is limited pathology; however, due to the normally high abundance of these cestode parasites within their hosts, they can be highly pathogenic (Abbou 1958). *Davainea proglottina* was the most abundant species found within all infected Galliformes. Hosts showed an aggregated distribution of mostly gravid proglottids within the small intestine. The life cycle of *D. proglottina* is relatively short. The common intermediate host of *D. proglottina* is a slug; cysticercoids are taken up by the Galliformes after digesting slugs. It takes approximately 8 days for the cysticercoids to fully develop after ingestion (Chandler 1923).

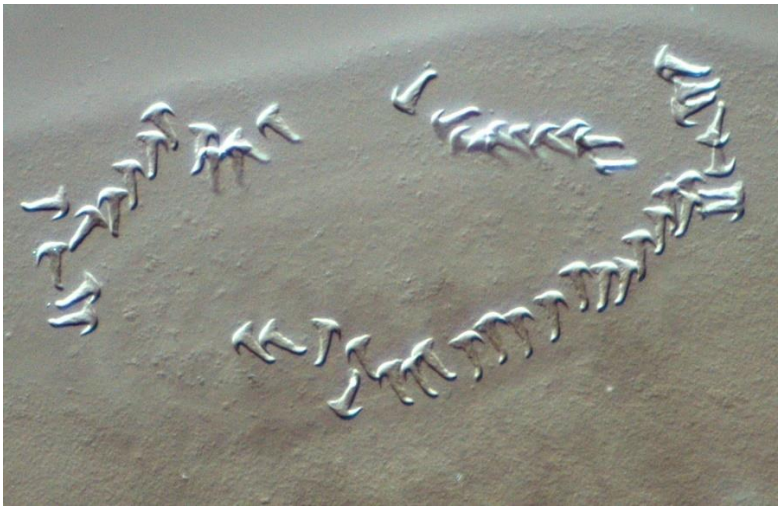

Figure 2. Rostellar hooks of *D. proglottina*

Hymenolepididae, is one of the most diverse families within the Cestoda class, Cyclophylidea order. Most tapeworms found within birds and mammals belong to the Hymenolepididae family. Their most defining feature is a single compact, postovarian vitelline gland. Hymenolepididae can have a direct or indirect lifecycle. The direct lifecycle is transmitted via fecal-oral route. The indirect lifecycle follows a "food-chain" type of transmission. Hymenolepididae has four major life cycle stages that include the egg, oncosphere, metacestode, and adult tapeworm. The metacestode stage infects the intermediate hosts, most commonly an arthropod. The adult tapeworm infects the definitive hosts after ingestion of the intermediate host.

Leucochloridium variae, a digenean found in the class Trematoda is well known for infecting Galliformes in North America. A defining morphological characteristic of *L. variae*, is the Distome body shape. Digeneans with the distome body form are known to have an oral and ventral sucker; both suckers can be seen clearly on Figure 3 Galliformes are nonmigratory birds; studies show the immature stages of *L. variae* are found within local mollusks (snails) Babero (1953). *Leucochloridium variae*

metacercaria live within the tentacles of snails where they look like maggots that attract different bird species. A study has found Galliforms at an early age, 1-6 days old, are more commonly infected with *L. variae* metacercaria (Lewis 1974). The adult stages of *L. variae* most commonly live within the cloaca of Galliformes, the definitive host (Lewis 1974). In this present study, *L. variae* showed an aggregated distribution where some of the collected Galliformes were infected with little to no adult *L. variae* while others were infected with hundreds.

Figure 3. *Leucochloridium variae*

Brachylaima fuscata, a digenean found in the class Trematoda. *Brachylaima fuscata* is entirely endoparasitic and well known for infecting vertebrates, including wild game and poultry (Heneberg et al. 2016). A defining morphological characteristic of *B. fuscata,* is the Echinostome body shape, i.e. presence of a collar of spines around the oral sucker. This body shape is unique due to the tandem, linear testes and large ventral suckers, as you can see in Figure 4. *Brachylaima fuscata* has a complete indirect lifecycle. The indirect lifecycle follows a "food-chain" type of transmission. *Brachylaima fuscata* has four major life cycle stage that include the egg, miracidium, cercaria, and adult. The egg is released into the water where it matures into miracidium.

The miracidium are ingested by the first intermediate host, almost always a snail, and mature into a mother sporocyst, which generate daughter rediae through asexual, daughter rediae produce free living cercaria. The cercaria then penetrate the second intermediate hosts, commonly known to be aquatic insects. The cercaria are then ingested by the definitive hosts, in our case would be the Alaskan Galliforms.

Figure 4. *Brachylaima fuscata*

Ascaridia compar, a nematode from the family Ascaridiidae, is one of the most common intestinal nematodes. Male ascaris are normally relatively smaller than females measuring 48-51 mm in length while females measure 19-49 mm in length (Tanveer et al. 2013). Males can also be distinguished from females by the presence of the copulatory bursa, a seen in Figure 5. A defining characteristic of the Ascaris family is the presence of three lips on the anterior end, as seen in Figure 6. Nematodes in the Ascaris family can experience direct or indirect lifecycles. The direct lifecycle is transmitted via fecal-oral route. The indirect lifecycle begins when eggs are passed in feces of an infected vertebrate hosts. The egg will go through two molt cycles before developing into the infective state. The larvae migrate onto grass where they are then ingested by a vertebrate host. It is important to note that the eggs are able to live within the soil for up to ten years.

Figure 5. Posterior end of male *Ascaridia compar*

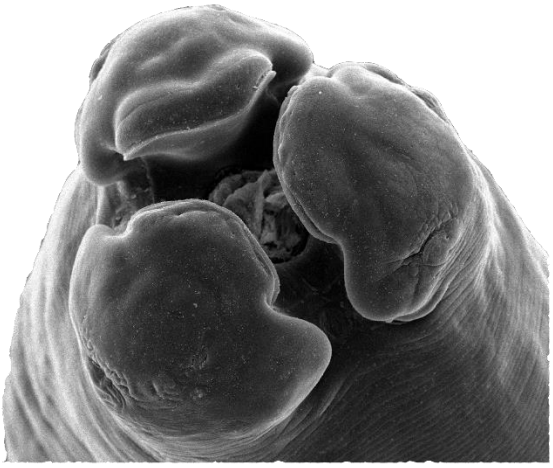

Figure 6. Anterior end of male *Ascaridia compar*

Capillaria spp., a nematode from the family Trichinellidae, is known for causing significant harm to its host when heavy infections are present. There is little known about the life cycles of these nematodes. *Capillaria spp.* commonly have direct lifecycles where the host becomes infected after ingesting feces from contaminated areas (Permin et al. 2010). *Capillaria spp.* are most commonly found within the small intestine of their hosts (although, some mammal capillariids are found in the stomach, esophagus,

and urinary bladder); however, in this study all adult worms collected were located in the large intestine.

Table 1. Summary of the data found on helminth species living within 83 ground dwelling birds.

Species: No. of each						
Helminth		Spruce Grouse 3	Rock ptarmigan 23	Willow ptarmigan 46	No. infected of total	Percent infected of total
No. infected						
Nematoda:						
	Ascaridia compar		3	2	5	6.9
	Capillaria sp.			7	7	9.7
Trematoda:						
	Leucochloridium variae	1	1	16	18	25
	Brachylaima fuscata		4	16	20	27.8
Cestoda:						
	Davainea proglattina	1	12	37	50	69.4
	Hymenolepidae sp.		8	1	9	12.5

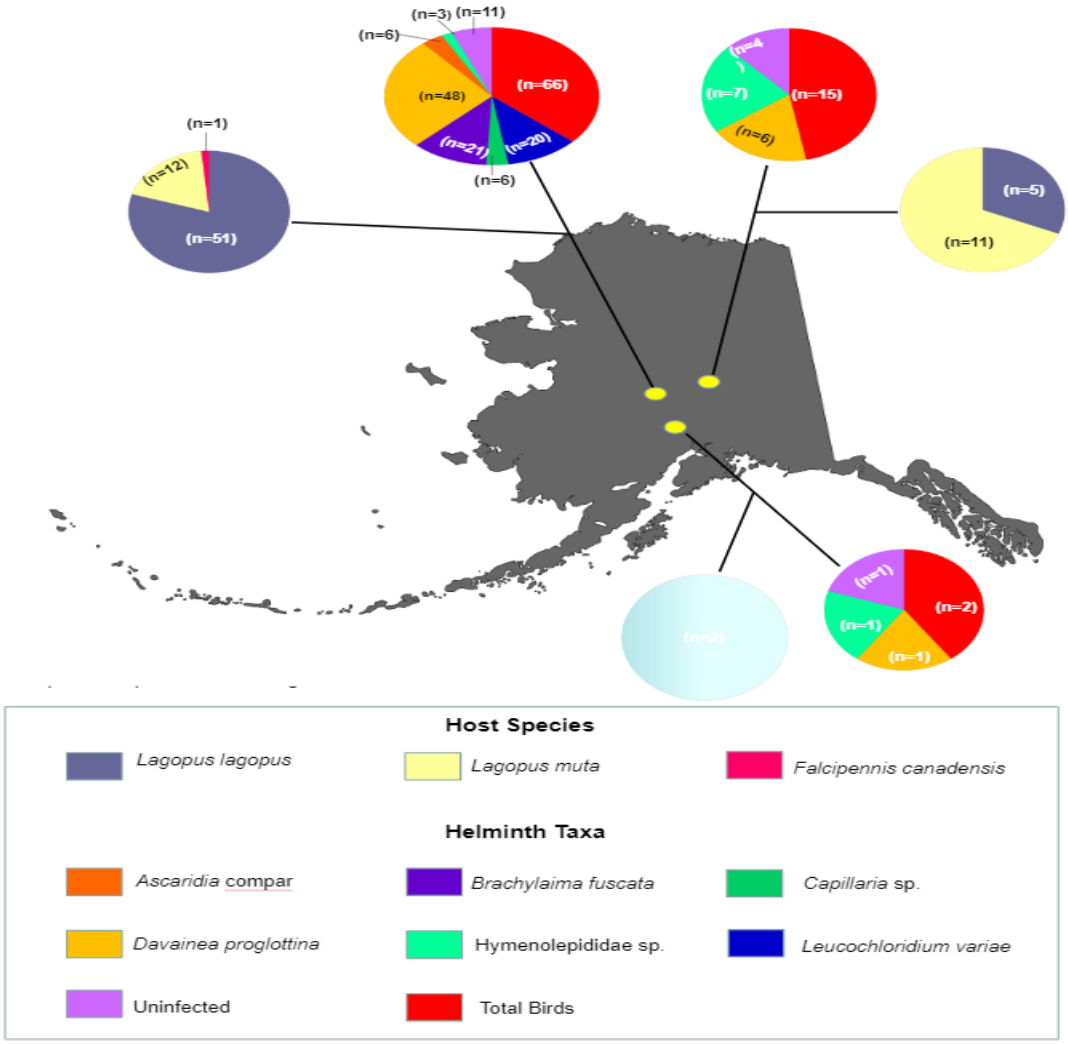

Figure 7. Alaskan map showing three main collection sites. Each site depicts the species of birds collected with also the species of parasites infecting said birds.

Out of a total of 564 blood and tissues samples tested, by the use of a TaqMan probe, 50 gallids tested positive for microfilaria (Table 2.). Figure 9 depicts the main collection site for blood and tissue samples, including the species of gallids collected at each site. The spruce grouse, *F. canadensis*, showed the highest prevalence of microfilaria; however, this could have been due to the larger sample size of this host. *F. canadensis* showed the highest prevalence of microfilaria at 25% of birds screened being

infected. *T. phasianellus* had the second highest prevalence of microfilaria at 16.70% of birds screened being infected. *L. muta* had a prevalence of 3.70% of birds screened being infected. *L. lagopus* had a prevalence of 3.1% of birds screened being infected. Figure 10 shows the presence or absence of microfilaria infecting the gallids from the main collection areas.

Filarial nematodes are vector-borne and experience an indirect life cycle. Most parasites produce eggs during the reproductive cycle; however, filarial nematodes do not lay eggs. Instead the female nematode produces microfilariae. These microfilariae are found in the blood and tissues of their host and remain there while maturing through two molts until eventually ingested by the arthropod vector, which in this study would be *Ornithomya fringillina*. Microfilaria are considered to be in the infective stage while present in vector.

It is important to note that when using 5µl of DNA the real-time PCR reaction failed; however, when using a smaller amount of DNA, 3µl of DNA, the reaction worked perfectly.

Table 2. Summary of data found on the presence of microfilaria found in Alaskan gallids

Species : No. of each	Location	Presence/Absence of microfilaria	Percent infected of total
Bonasa umbellus *23*	Tanana, Baker, Nenana, Fairbanks, Donnelly	0	
Dendragapus fuliginosus *8*	Lemesurier Island	0	
Falcipennis canadensis *139*	Nenana, Fairbanks, Sterling, Copper Landing, Crown Point, Portage, Wasilla, Susitna, Galena, Wiseman,	36	25.90%
Lagopus lagopus *223*	Prudhoe Bay, sagwon, Newhalen, Port Alsworth, Cold Bay, Sunrise, Paxon, Miller House, Bettles.	7	3.10%
Lagopus lecura *23*	Port Alsworth, Anchorage, Wasilla, Palmer	0	
Lagopus muta *136*	Wiseman, Prudhoe Bay, Miller House, Wortmanns, Glacier View, Nelchina, Nome	5	3.70%
Tympanuchus phasianellus *12*	Nenana, Fairbanks, Nikolai	2	16.70%

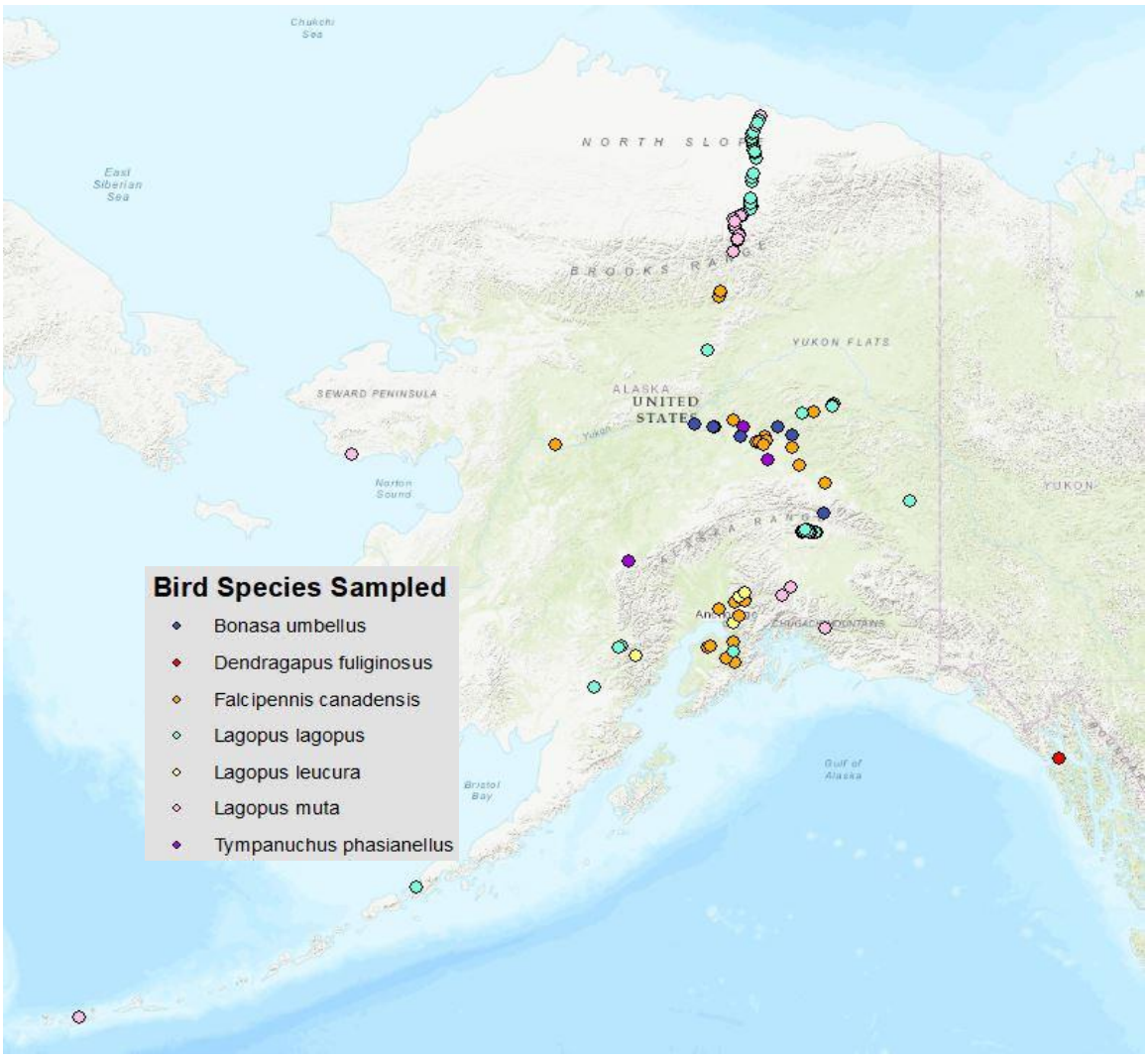

Figure 9. Alaskan map showing main collection sites for blood/tissue samples. Each site depicts the species of birds collected.

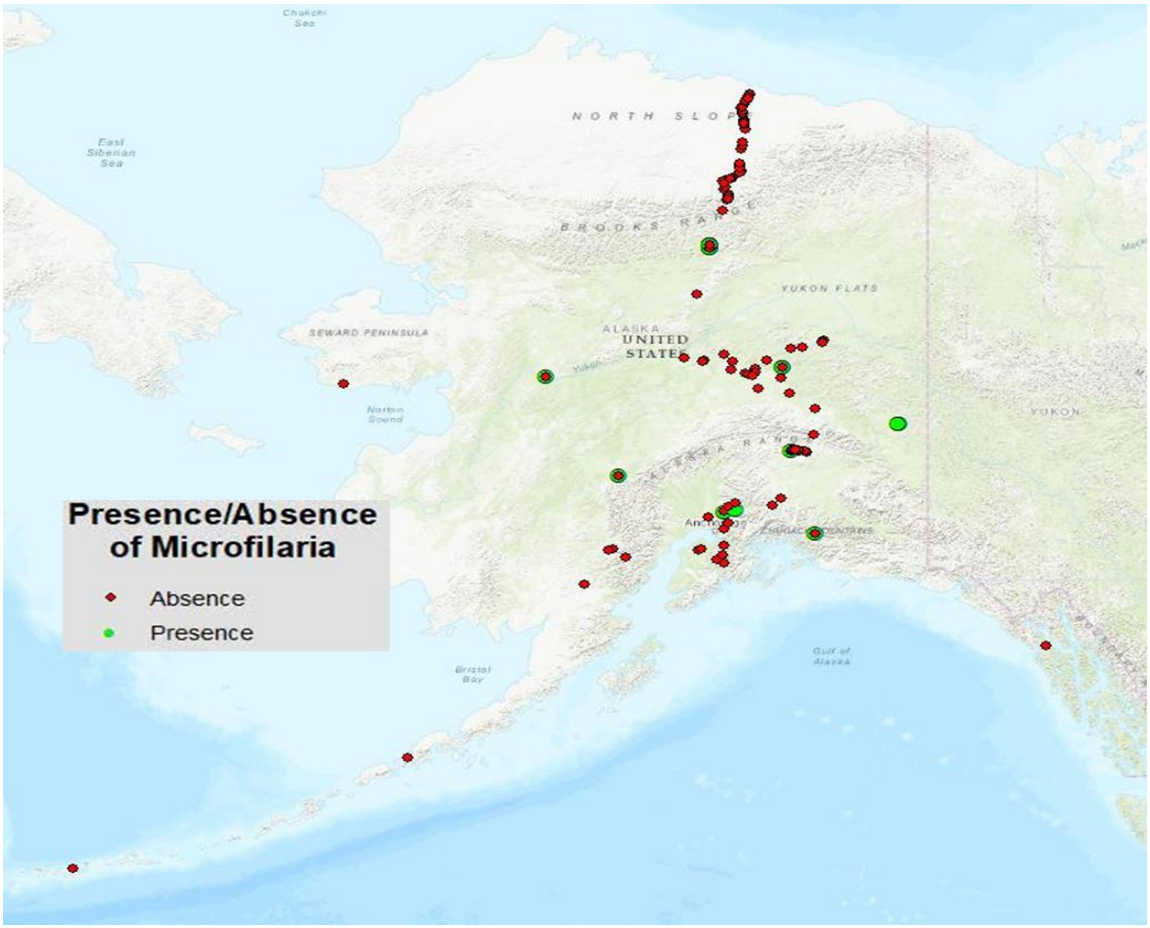

Figure 10. Alaskan map showing main collection sites of blood/tissue samples. Each site depicts the species of birds collected with also the presence and absence of microfilaria infecting said birds.

Table 3. Summary of data found on flies dwelling on Alaskan gallids

Species: No. of each	Location	No. of flies screened	No. of total infected	Percent infected of total
Bonasa umbellus	Baker, Woodchopper	16	0	0
Falcipennis canadensis	Buffalo Soapstone	4	1	25%
Lagopus lagopus	Paxson	1	0	0
Lagopus muta	Millerhouse	1	0	0

Out of 22 Hippoboscid flies (parasitic on birds) screened for the presence of microfilaria (Table 3) one tested positive, a fly found from the bird species *F. canadensis*. A 428 bp fragment of the COI mtDNA gene amplified for the infected fly matched 100% the sequence of *Ornithomya fringillina*. This positive sample gives strong evidence that *Ornithomya fringillina* may be the vector for the species of *Splendidofilaria* infecting birds in our study.

Discussion

This was the first molecular survey of helminths from Alaskan ptarmigan and grouse. We provide the first 18S and 28S DNA sequences of *B. fuscata, D. proglottina,* Hymenolepididae sp., *L. variae and first* 12S *and* COI DNA sequnces from *A. compar, Splendidofilarinae* sp. and *Capillaria* spp. Sequence data collected will allow for more robust phylogenetic analyses of our newly sequenced taxa and already available sequenced taxa.

This study will help build baseline data related to helminth diversity, geographic distribution, and host/parasite relationships for AK gallids. Such baselines will allow for future studies predicting/measuring the impact of global environmental change on avian helminth distribution and host associations. Greater morphological examination of helminth taxa will be done for better species identification. Taqman real-time PCR assay will allow for more reliable results when screening blood and tissue samples. Future work will focus on a greater number of birds.

During this study we found that 89% of all samples collected were infected with one or more parasites. Parasite species from the Nematoda and Platyhelminthes phyla

were collected from the host samples. Four species collected were identified to species level using morphological and molecular techniques; however, two taxa we were only able to narrow down to family and genus level, Hymenolepididae *spp. and Capillaria spp.* Future research will focus on the phylogeny of the Hymenolepididae *spp. and Capillaria spp.* to further our knowledge, understanding, and give us the opportunity to input these species' DNA sequences into GenBank in order to help others.

This study also provided the first usage of a Taqman PCR assay to screen for the presence of *Splendidofilaria* spp. Previous techniques required the use of peripheral blood smears to screen for microfilaria. This technique was not only unreliable, but it was also extremely time consuming. By using the Taqman PCR assay we yield positive results from living or deceased microfilaria present in blood and/or tissue samples in a timely manner. In this study we also used the Taqman PCR assay to screen flies harboring the samples collected for the presence of microfilaria in order to identify the possible vector.

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